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論　文　の　内　容　の　要　旨

The inherent plasticity and multi-lineage potential of stem cells make them promising cell sources for tissue engineering and regenerative medicine. However, controlling over behaviors and functions of stem cells, such as proliferation and differentiation, is extremely required to fully realize the therapeutic potential of stem cells.

Either *in vivo* or *in vitro* stem cell functions are regulated by intricate reciprocal interactions between cells and their microenvironment. Stem cell behaviors are highly sensitive to the biochemical and physical factors from surrounding microenvironment such as the extracellular matrix (ECM), neighboring cells, and surface properties. Due to the limitation of current technology to track *in vivo* cells, it is still very difficult to directly investigate cell behaviors *in vivo*. Accordingly, *in vitro* cell culture is the indispensable approach for stem cell research. Nonetheless, the conventional cell culture surface is difficult to precisely control cell microenvironment, such as the location, size, and shape of individual cells, contact between cells, and to directly compare the effects of various microenvironmental factors, such as biochemical compositions, topographical features, wettability, electric charges, and stiffness of local surface. In contrast, micropatterning techniques enable the precise control of most of the important factors from cell microenvironment by modifying the physicochemical properties of cell-culture substrates at predefined locations and sub-cellular scales. Therefore, the effect of individual environmental factor on cell function and fate can be accurately assessed.

In this study, we integrated a simple and robust micropatterning method with the stem cell research on the purpose of manipulation of stem cell functions. Various micropatterns with particular features were designed by using different photomasks to investigate the pivotal components of cell microenvironment in a highly controllable manner. Photo-reactive poly(vinyl alcohol) (PVA) were synthesized by coupling PVA with UV-sensitive azidophenyl group and locally grafted to the commonly used cell-culture polystyrene (PSt) surface by UV irradiation through photomasks. PVA was chosen as non-fouling polymer due to its superior ability to resist protein adsorption and cell adhesion. Long-term cell culture at either multiple- or single-cell level was realized on the PVA-micropatterned polymer surfaces because the

micro-patterned PVA hydrogel was very stable and highly hydrated in the aqueous culture medium. Cell density, spreading, and shape of human bone marrow-derived mesenchymal stem cells (MSCs) were regulated by the ratio of PSt area to PVA area, size, and geometry of the micropatterns, respectively. The effect of cell density, spreading area, and cell shape on the functions of MSCs such as proliferation and differentiation were directly compared on the PVA-micropatterned PSt surface. Moreover, PVA-poly(acrylic acid) (PAAc)-micropatterned PSt surface was prepared to study the electrostatic effect on the functions of individual MSCs with highly controlled and uniform cell shape.

(1) Manipulation of cell density and its effect on osteogenic and chondrogenic differentiation of MSCs

Photo-reactive PVA was synthesized by coupling the hydroxyl groups of PVA with 4-azidobenzoic acid. The introduction of photo-reactive azidophenyl groups in PVA was confirmed by the appearance of peaks assigned to the azidophenyl proton around 7 and 8 ppm in the ^{1}H-NMR spectrum. The percentage of the hydroxyl groups in PVA coupled with the azidophenyl groups was 2.1%. To control different cell densities on a single surface, a micropatterned surface with different area ratios of cell non-adhesive PVA to cell-adhesive PSt regions was prepared using photo-reactive PVA by UV photolithography. MSCs were cultured on the micropatterned surface. Cells moved from non-adhesive PVA regions to cell-adhesive PSt regions and a gradient pattern of cell density (2.6-112.5 $\times$ 10^3 cells/cm^2) was generated. The effect of cell density on proliferation and osteogenic and condrogenic differentiation of MSCs was investigated by using the gradient cell density pattern. MSCs at a low density showed higher proliferation than the cells at a high density. Osteogenic differentiation of MSCs was analyzed by alkaline phosphatase (ALP) staining and expression of osteogenesis-specific genes (*ALP* and *BSP*). Although MSCs at both low and high densities showed osteogenic differentiation, high cell density initiated faster osteogenic differentiation than low cell density. Immunocytochemical staining of type II collagen was carried out to examine the effect of cell density on chondrogenic differentiaton of MSCs. The results showed that high cell density was required to induce chondrogenic differentiation of MSCs. Therefore, this micropatterning method enabled convenient manipulation of cell distribution in a density gradient manner for the direct and systematic investigation of stem cell functions related to cell density.

(2) Manipulation of cell spreading and its effect on osteogenic and adipogenic differentiation of MSCs

To control different degree of cell spreading with the same cell shape, a series of circular cell-adhesive PSt micropatterns with different diameters were created. The PSt micropatterns were surrounded by non-adhesive PVA regions. The diameters of the PSt circles in the micropatterns were 40, 60, and 80 μm. MSCs were cultured on the micropatterns. MSCs only adhered on the PSt circles and most of the PSt circles were occupied by a single cell. Therefore, the heterogeneity of cell population in routine cell culture was reduced, and cell spreading and differentiation of individual MSCs were investigated on the micropatterned surface. F-actin of MSCs was stained with Alexa Fluor® 488 phalloidin and cell nucleus was counterstained with 4', 6-diamidino-2-phenylindole (DAPI). As shown in Figure 3, F-actin staining revealed that the spreading of MSCs followed the underlying PSt circles and was confined by the surrounding non-adhesive PVA regions, whereas MSCs spread freely on bare PSt surface (non-pattern). The assembly and organization of actin filaments were regulated by the degree of cell spreading. Individual MSCs with the largest degree of spreading (80 μm) mainly assembled actin in the radial and concentric directions of the circle. However, such organization of the actin structure weakened as the degree of cell spreading decreased. On the smallest circle (40 μm), MSCs predominately assembled actin along their edges, between the cell-adhesive PSt and non-adhesive PVA. The MSCs cultured on non-pattern surface did not exhibit any regular organization. Furthermore, osteogenic and adipogenic differentiation of MSCs on the micropatterns were compared by ALP and Oil Red O staining, respectively.

The probabilities of adipogenesis and osteogenesis at different cell spreading areas were studied by calculating the percentage of MSCs that committed to an adipocyte or osteoblast lineage. MSCs positively stained by Oil Red O or ALP were considered to be adipocytes or osteoblasts, and only single cells on each circle (as confirmed by cell nuclear staining) were counted. The results indicate that the differentiation of MSCs was dependent on the degree of cell spreading. Increased cell spreading facilitated the osteogenic differentiation but suppressed the adipogenic differentiation of MSCs.

(3) Manipulation of cell shape and its effect on adipogenic differentiation of MSCs

To control different cell shapes with the same degree of cell spreading, cell-adhesive PSt micropatterns of equilateral triangular, square, pentagonal, hexagonal, and circular geometries surrounded by cell non-adhesive PVA regions were prepared by micropatterning photo-reactive PVA with photomask. These different geometries had the same surface area for cell spreading (1,134 μm^2). Human MSCs were cultured on the micropatterned surface and different cell shapes were manipulated by the geometries of PSt micropatterns. The distribution patterns of MSCs actin filaments were similar among these cell shapes, that is, actin filaments were predominately assembled along the peripheral edges of cell profile. In contrast, the MSCs freely spread on the non-pattern surface and irregularly aligned actin filaments and stress fibers. Moreover, the organization and assembly of F-actin weakened and remolded during MSC adipogenesis, particularly the actin filaments at the edges between the adhesive PSt and the non-adhesive PVA. The effect of different cell shapes on the probability of MSCs adipogenesis was studied by calculating the percentage of MSCs that committed to an adipocyte lineage and by analyzing the area of positively stained lipid vacuoles. Cells containing lipid vacuoles that were positively stained by Oil Red O were considered as adipocytes, and only single cells from each shape were counted. According to the lipid vacuoles staining result, adipogenic differentiation potential of MSCs was similar on the triangular, square, pentagonal, hexagonal, and circular micropatterns. The triangular, square, pentagonal, hexagonal and circular shapes did not have a significantly different effect on the adipogenic differentiation of MSCs.

(4) Preparation of micropattern of negatively charged polymer and its effect on MSCs functions

Circular micropatterns of negatively charged poly(acrylic acid) (PAAc) and neutral PSt were created with different diameters using UV photolithography. These micropatterns were surrounded by non-adhesive PVA regions. The diameters of the circular PAAc and PSt micropatterns were 40, 60, and 80 μm. The PVA-PAAc-micropatterned surface was stained with Brilliant Green solution to show the PAAc micropatterns. The basic dye interacted electrostatically with the negatively charged PAAc, and the circular PAAc micropatterns were visualized by a dark green color under optical microscopy. During the staining process, the dye permeated through the overlying PVA layer to the PAAc layer below, causing the surrounding light green color. These micropatterned surfaces were used to control the shape and spreading of stem cells and to investigate the electrostatic effect derived from different chemical groups on the functions of individual and multiple human MSCs. The assembly and distribution of actin filaments of individual MSCs correlated with the degree of cell spreading as well as the surface charge of the underlying substrate. On 40 μm micropatterns, the distribution of actin filaments was homogeneous on the negatively charged PAAc circles, whereas the circular MSCs primarily organized actin filaments along the periphery of cell on the neutral PSt circles. The organization of the actin structure became more ordered as the degree of cell spreading increased (60 μm micropatterns). As the cell spreading further enlarged (80 μm micropatterns), the radial and concentric arrangement of actin filaments appeared on both PAAc and PSt circles. Cell division and proliferation were evaluated by comparing the percentages of PAAc and PSt micropatterns that were occupied by single cells. The proliferation of MSCs on the PAAc and PSt micropatterns was not obvious and was independent of the circular size after 24 hours. However, MSCs divided and proliferated during the adipogenic culture period. Moreover, the degree of MSC adipogenesis was

evaluated by comparing the percentage of PAAc and PSt micropatterns that contained Oil Red O stained cells. At the single-cell level, the adipogenesis of MSCs was enhanced on the negatively charged PAAc micropatterns when the circular diameter was small (40 and 60 μm micropatterns). By contrast, the adipogenesis of MSCs was similar between the negatively charged PAAc and the neutral PSt micropatterns when the circular diameter was large (80 μm micropatterns). Moreover, the percentage of differentiated MSCs decreased as the circular diameter increased; this trend was independent of surface charge. At the multiple-cell level, the negatively charged PAAc micropatterns promoted the adipogenesis of MSCs when compared to the neutral PSt micropatterns. However, the effect of the circular diameter was not evident in the adipogenesis of groups of multiple MSCs.

In conclusion, diverse micropatterns were prepared on commonly used cell-culture PSt surface using photo-reactive polymer and UV photolithography. The respective effect of cell density, spreading, shape, and surface charge on the functions of bone marrow-derived MSCs was investigated. This simple micropatterning method using photo-reactive polymers is advantageous for fabricating arbitrary micropatterns on prevalent cell-culture substrates, and cell functions can be directly and systematically investigated on a single surface without external interferences resulting from separate cell culture and pre-coated cell-adhesive molecules. Moreover, it enables the analysis of entire cell populations at both single- and multiple-cell levels, significantly facilitating the identification of the optimal parameters to manipulate the functions of stem cells. The insights derived from this study will be useful to design suitable biomaterials for stem cell research and scaffolds for tissue regeneration.

審 査 の 結 果 の 要 旨

本論文では、マイクロパターン化表面を利用することによって、骨髄由来の間葉系幹細胞の機能の制御を試みたものである。光反応性のポリビニルアルコールを合成し、フォトリソグラフィー法により、細胞培養用ポリスチレン基板の表面に、細胞接着領域と非接着領域の面積比が異なるパターンや、細胞接着領域の大きさ・形状が異なるマイクロパターンの構造を作製した。これらの基板を用いて幹細胞を培養し、細胞密度を勾配的に変化させた多細胞アレイ、および細胞の伸展面積と形状が異なる単細胞アレイを形成し、細胞の密度勾配と伸展面積および形状が幹細胞の軟骨、骨と脂肪細胞への分化に与える影響について調べた。その結果、細胞密度が増加するにしたがって軟骨分化は促進され、骨分化も早期に誘導されることがわかった。また、細胞の伸展面積の増加にともない骨芽細胞への分化が促進されたが、一方、脂肪細胞への分化は抑制されることがわかった。さらに、負電荷の表面はポリスチレン表面に比べて幹細胞の脂肪分化を促進することを見い出した。細胞の形状による幹細胞の脂肪細胞への分化への影響は観察されなかった。このように、マイクロパターン化した表面をもつ培養材料は、幹細胞の単一細胞および多細胞レベルでの機能を同一の表面で比較するのにきわめて有用であることが示された。得られた成果は、再生医療のための幹細胞の基礎研究および足場材料の設計に役立つ知見を与え、本研究分野の学術的貢献に資することが期待される。よって、本論文は博士（工学）の学位論文として価値あるものと認める。

平成 24 年 2 月 10 日、数理物質科学研究科学位論文審査委員会において審査委員の全員出席のもと、著者に論文について説明を求め、関連事項につき質疑応答を行った。その結果、審査委員全員によって、合格と判定された。

上記の論文審査ならびに最終試験の結果に基づき、著者は博士（工学）の学位を受けるに十分な資格を有するものと認める。